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ISOLATION AND CHARACTERIZATION OF HUMAN SKIN LYSOZYME*

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ABSTRACT

Lysozyme was isolated from human skin by extraction with 0.1 M acetate buffer, pH 4.5 containing 0.1 M KCl. Crude enzyme was purified by the method of three successive chromatographic processes on Amberlite IRC-50, carboxymethylcellulose, and Amberlite IRC-50. The purified human skin lysozyme was determined to be homogeneous by polyacrylamide gel electrophoresis. Molecular weight of the enzyme is estimated to be approximately 15,000 either by dialysis with calibrated membranes or by gel filtration through Sephadex G-50 column. The amino acid composition and several biophysical properties of human skin lysozyme were found to be very similar to those of human milk and leucocyte lysozymes.

The occurrence of the lytic enzyme, lysozyme or muramidase, in the skin was recognized by Liss (1). The lysozyme content of homogenates of hairless mouse skin and extracts of human skin surface have been measured by Klenha and Krs (2). Ogawa, one of the authors, has obtained both epidermis and dermis from human skin separated mechanically and has determined their lytic activity. More lytic activity was present in the epidermal than in the dermal portion. Also, he found the lysozyme content of human skin to be 60–120 μg per wet g of skin (3).

Jollés *et al.* purified a great number of lysozymes from normal human tissues and secretions, *i.e.* from milk (4, 5), tear (5), saliva (6), placenta (7), spleen, serum and leucocytes (8). Human serum and urinary lysozymes in monocytic and monomyelocytic leukemias were also purified by Osserman and Lawlor (9).

In the present study, human skin lysozyme was purified and characterized with respect to activity, stability, and amino acid composition.

MATERIALS AND METHODS

Preparation of human skin lysozyme. Skin was obtained from the abdominal region of a cadaver within 24 hours after death and frozen at −20° C. It was used within two weeks, and the original activity remained stable during storage.

The frozen specimen (100 g) was washed at 4° C with physiological saline for removal of fat and blood. It was finely minced, and then homogenized mechanically with one liter of ice cold 0.1 M KCl-0.1 M acetate buffer, pH 4.5, in a Waring Blender for 15 minutes. The homogenate was submitted to centrifugation for 20 minutes at 6,000 × g in a refrigerated instrument. The sediment was extracted twice in the same manner. The supernatants were combined and filtered through cheese cloth. The cloudy filtrate was dialyzed against 0.01 M acetic acid, and centrifuged at 9,000 × g for 15 minutes. The supernatant which contained the lysozyme was immediately lyophilized and stored at −20° C.

The lyophilized powder was dissolved in 100 ml of 0.2 M phosphate buffer, pH 6.5. The insoluble materials were removed by centrifugation at 9,000 × g for 15 minutes. The clear supernatant was put directly on a column (3.5 × 20 cm) of Amberlite IRC-50 (200–400 mesh), which was equilbrated with the above buffer. Stepwise elution was carried out successively with 0.2 M phosphate buffer, pH 6.5, and 0.8 M phosphate buffer, pH 6.5, at a flow rate of 100 ml per hour. The fractions (5 ml) were monitored by the measurement of optical density at 280 mμ. The lytic activity was measured by the use of *Micrococcus lysodeikticus* cells. The lysozyme rich fractions were combined, dialyzed against the distilled water, and lyophilized. The lyophilized sample was dissolved in 10 ml of 0.01 M phosphate buffer, pH 5.9. It was put on a column (1.5 × 25 cm) of carboxymethylcellulose (Brown Co. 0.87 meq.), which was equilibrated with the same buffer. Gradient elution was performed using a first vessel containing 200 ml of 0.01 M phosphate buffer, pH 5.9 and a second vessel containing 200 ml of 0.2 M phosphate buffer, pH 7.2. The lysozyme fractions were combined, dialyzed against the distilled water, and lyophilized. The lyophilized sample was further dissolved in 1.0 ml of 0.2 M phosphate buffer, pH 6.98. It was put on a column of Amberlite IRC-50 (1.5 × 20 cm) previously equilibrated with the

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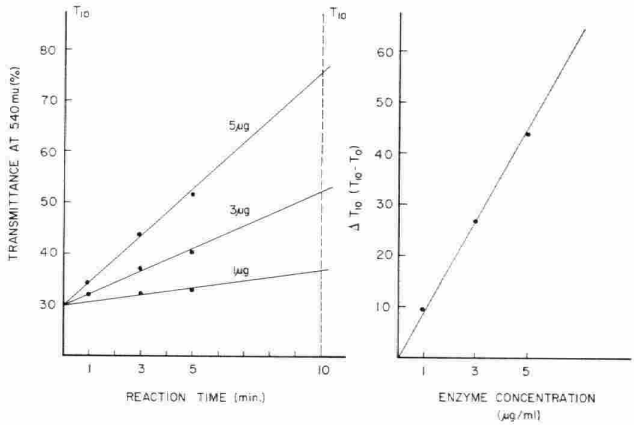

FIG. 1. Standard curve for enzyme activity of hen egg white lysozyme. Assay mixture: Micrococcus lysodeikticus cells in 0.067 M phosphate buffer pH 6.2 (0.3 mg/ml), 1.5 ml; 0.3 M NaCl-0.067 M phosphate buffer pH 6.2, 0.5 ml; Lysozyme (egg white lysozyme) in 0.067 M phospate buffer pH 6.2 (1, 3 and 5 μg/ml), 1.0 ml. T = Transmittance (T0, at zero time; T10, after 10 min of incubation).

same buffer. Elution was carried out with 0.2 M phosphate buffer, pH 6.98, at a flow rate of 20 ml per hour. The lysozyme fractions were combined, dialyzed against the distilled water, and lyophilized. The purified preparations were stored at −20° C.

The human milk and leucocyte lysozymes were prepared according to the method described by Kimura *et al.* (10).

Assay of lysosyme activity. The lytic activity of the lysozyme was determined by the method of Parry *et al* (11) with slight modification; lyophilized cells of *Micrococcus lysodeikticus* were suspended into 0.067 M phosphate buffer, pH 6.2 containing 0.075 M NaCl (0.225 mg/ml). The mixture was preincubated for 3 minutes at 35° C before use. Aliquots of 0.1 ml of each fraction obtained from the column chromatography were diluted with 0.067 M phosphate buffer, pH 6.2 and 1.0 ml of this diluted solution was added to 2.0 ml of the assay mixture after preincubation for 3 minutes at 35° C. Then, the reaction mixture was incubated for 5 minutes at 35° C. The decrease in turbidity at 540 mμ was measured at 1, 3 and 5 minutes during which period the ΔT/min remained constant (cf 11) and the decrease turbidity for 10 minutes (ΔT/10 min) was extrapolated from the plot of ΔT *vs* time at this early time. A standard curve was made by the use of hen egg white lysozyme, as shown in Figure 1. Lysozyme activity was expressed relative to the lytic activity of hen egg white lysozyme. The hen egg white lysozyme (×6 recrystallized Lot. No. 4211) was obtained from Seikagaku Kogyo Co., Tokyo.

Determination of optimum pH, ionic strength and preincubation on the enzyme activity. The lysozyme activity was measured either in 0.067 M acetate buffers, pH 4.0 to 5.0 or in 0.067 M phosphate buffers, pH 5.5 to 7.5. The suspension of *Micrococcus lysodeikticus* cells in distilled water (300 mg/100 ml) was mixed with 0.1 M acetate buffer or 0.1 M phosphate buffer at a ratio of one to nine in volume. One milliliter of 0.3 M aqueous NaCl was added to 3.0 ml of the cell suspension. After the preincubation of the mixture for 3 minutes at 35° C, 0.1 ml of the lysozyme aqueous solution (1 to 3 micrograms) was added to the mixture. Then, the reaction mixture was incubated at 35°C. The decrease in turbidity at 540 mμ was measured at 1, 3 and 5 minutes and the ΔT/10 min was extrapolated.

The lysozyme activity was measured at various concentrations of NaCl. Aliquots of 1.0 ml of NaCl at various concentrations were added to 3.0 ml of the *Micrococcus lysodeikticus* cell aqueous suspension (0.3 mg/ml). After the preincubation of the mixture for 3 minutes at 35° C, 0.1 ml of the lysozyme solution (1 to 3 micrograms) was added to the mixture. The activity was measured by the method as described above and it was expressed as ΔT/10 min.

Lysozyme solution (1 to 3 micrograms) in 0.5 ml of 0.8% NaCl-0.02 M phosphate buffer, pH 7.5, was preincubated for different periods of time at 37° C. The solution was then added to 3.0 ml of *Micrococcus lysodeikticus* cell suspension in 0.05 M NaCl-0.067 M phosphate buffer, pH 6.2, incubated for 3 minutes at 35° C. Then, the activity was measured by the method as described above.

Amino acid analysis. Lysozyme (approximately 0.5 mg) was hydrolyzed with 6N HCl at 110° C for

24 hours *in vacuo*. The hydrolysate was analyzed in an amino acid analyzer (Hitachi amino acid analyzer, Model 034, expanded system). Cystine and cysteine were estimated as cysteic acid after the performic acid treatment. Tryptophan content was measured spectrophotometrically. Molar ratios were determined by analyzing a standard amino acid mixture.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of lysozyme was performed according to the method of Reisfeld *et al.* (12). Twenty-five to 50 μg of lysozyme per one gel column was moved in the buffer, pH 4.3, for 2 hours by 3 mA of current. The protein bands were stained with amido black dye.

RESULTS

From 100 g of the human skin, 0.3 to 0.9 mg of lysozyme was obtained after purification by the three chromatographic processes. The lysozyme rich powder prepared from the skin homogenate was chromatographed on Amberlite IRC-50, as shown in Figure 2 (A). The lysozyme fraction was further chromatographed on carboxymethylcellulose, as shown in Figure 2 (B). The main lysozyme fraction was finally chromatographed on Amberlite IRC-50, as shown in Figure 2 (C). A small peak was eluted in front of the main peak on the final Amberlite IRC-50 chromatography. This sub-fraction of lysozyme was not analyzed for biochemical properties and amino acid composition. The overall yield of lysozyme was approximately 5–7.5% for the original lysozyme activity.

An amount of 2.5 mg of the purified human skin lysozyme was obtained from five preparations. Electrophoresis on acrylamide gel at pH 4.2 showed that the protein migrated in a single band at this pH.

The spectrum of a 0.282% aqueous solution of human skin lysozyme is presented in Figure 3. The extinction coefficient of a 1% solution at 280 mμ was found to be 25.9.

The amino acid composition of human skin lysozyme is shown in relation to hen egg white lysozyme (see Table). The relative molar ratio was calculated on the basis of two phenylalanine residues per molecule of human skin lysozyme. Results indicated that the amino acid composition of human skin lysozyme was similar to

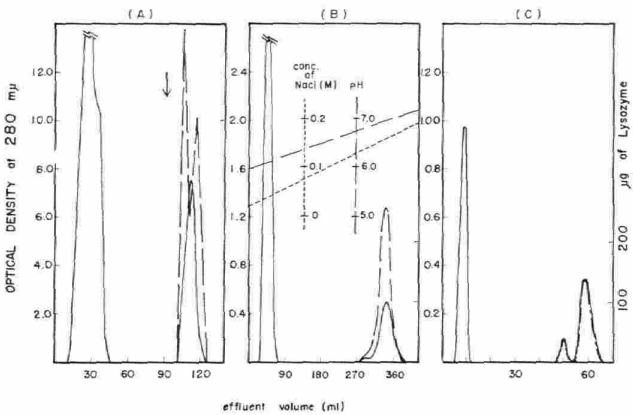

FIG. 2. Purification of human skin lysozyme by three chromatographic processes. (A) Column chromatography of extract from skin specimen on Amberlite IRC-50. The resin (column size; 3.5 × 20 cm), was equilibrated with 0.2 M phosphate buffer, pH 6.5. The elution was carried out stepwise with 0.2 M phosphate buffer, pH 6.5 and 0.8 M phosphate buffer, pH 6.5. (B) Column chromatography of lysozyme fraction from A on carboxymethylcellulose. The cellulose (column size; 1.5 × 25 cm) was equilibrated with 0.01 M phosphate buffer, pH 5.9. The elution was carried out with a linear gradient producing by 200 ml of 0.01 M phosphate buffer, pH 5.9 (the first chamber) and 200 ml of 0.2 M phosphate buffer, pH 7.2 (the second chamber). (C) Column chromatography of lysozyme fraction from B on Amberlite IRC-50. The resin (column size; 1.5 × 20 cm), was equilibrated with 0.2 M phosphate buffer, pH 6.98. The chromatography was carried out with the above buffer. Absorbancy at 280 mμ and lysozyme activity of fraction are indicated by linear line and dotted line respectively.

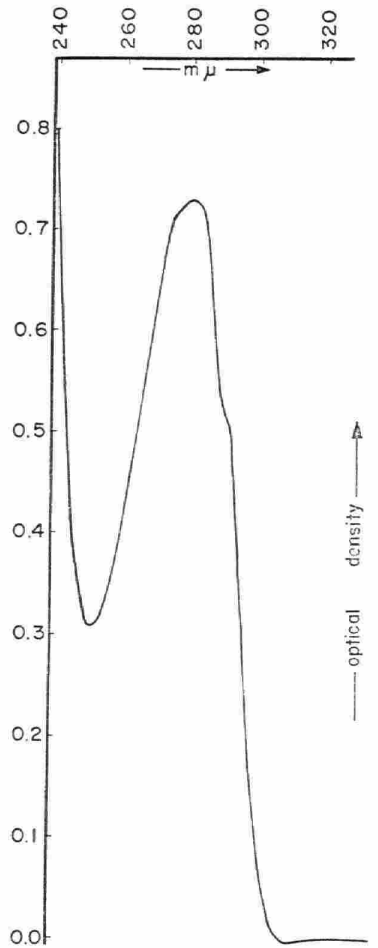

FIG. 3. Absorption spectrum of human skin lysozyme. Concentration of enzyme was 0.282%. Measurement was carried out at room temperature.

those of human milk and human leucocyte lysozymes.

When the lysozyme assays were performed by the standard procedure using a cell suspension of *Micrococcus lysodeikticus*, the lytic activity of human skin lysozyme was two to three times higher than that of hen egg white lysozyme.

The optimum pH of human skin lysozyme was found to be approximately pH 6, which is similar to the values for lysozyme from human milk and hen egg white, as shown in Figure 4.

To clarify the effect of the ionic strength on the lytic activity of three lysozymes, human skin and milk lysozyme and hen egg white lysozyme were examined. Each lysozyme was nearly inactive in the absence of NaCl. The maximum activity of the three lysozymes was demonstrated in the region of 0.05 to 0.1 M NaCl. They were increasingly inhibited as the concentration of NaCl was increased above 0.1 M (Fig. 5).

To determine the effects of preincubation at 37° C at pH 7.5 on the lytic activity of lysozymes, human skin and milk lysozymes and hen

TABLE

Amino acid composition of lysozymes from human skin, human milk and hen egg white

The relative molar ratio is calculated on the basis of two phenylalanine residues per molecule of human skin lysozyme. Threonine and serine residues are not corrected for destruction during the acid hydrolysis. Half cystine residues are calculated from the value of cysteic acid.

AMINO ACID COMPOSITION

amino acid	HenEggWhite Lysozyme	Human Lysozyme		
		H S L	H M L	H L L
Lys	6	5	5	5
His	1	1	1	1
Arg	11	11	11	11
Asx	Asp 8 Asn 13	18	18	17-18
Thr*	7	(4)	(4)	(4)
Ser*	10	(5)	(5)	(4)
Glx	Glu 2 Gln 3	8	8-9	8-9
Pro	2	2	2	2
Gly	12	11	11	11
Ala	12	12	12-14	12-14
1/2Cys**	8	8	8	8
Val	6	8	8	7-8
Met	2	2	2	2
Ile	6	4	4-5	4-5
Leu	8	7	7-8	7-8
Tyr	3	5	5-6	5-6
Phe	3	2	2	2
Trp	6	5	5	5

* not corrected

** Cysteic acid & CM-cystein

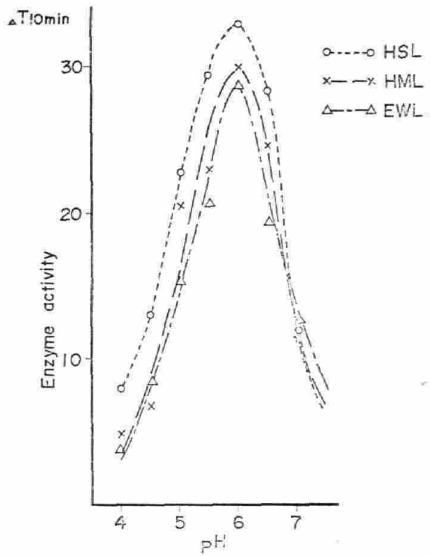

FIG. 4. Effect of pH on lysozyme activity. HSL: human skin lysozyme; HML: human milk lysozyme; EWL: hen egg white lysozyme.

egg white lysozyme were examined. Human lysozymes (skin and milk) demonstrated two periods of activation (at 30 and 180 minutes) and one of inhibition (at 90 minutes), but hen egg white lysozyme demonstrated only activation; one strong activation at 30 minutes and the other weak activation at 180 minutes. These results are shown in Figure 6.

DISCUSSION

Skin lysozyme was purified in satisfactory yield from human skin specimens by extraction with acidic buffer, followed by chromatography on Amberlite IRC-50, carboxymethylcellulose, and Amberlite IRC-50 in succession. The purified lysozyme preparation was found to be homogeneous by polyacrylamide gel electrophoresis.

The absorption spectrum of human skin lysozyme was very similar to that of human milk lysozyme as reported by Jollés *et al.* (5). The extinction coefficient of human skin lysozyme in 1% solution at 280 mμ was determined as 25.6, which is near to that reported by Shahani *et al.* (13).

The molecular weight of human skin lysozyme was estimated to be about 15,000 either by dialysis using calibrated cellophane membranes or by gel filtration through Saphadex compared with myoglobin (M. W. = 17,800) and cytochrome C (M. W. = 12,400). This molecular weight estimation of human skin lysozyme is consistent with that of human milk lysozyme. Moreover, the amino acid composition of human skin lysozyme reported here is not significantly different from those of human milk and leucocyte lysozyme, although they are markedly different from that of hen egg white lysozyme.

The effects of pH and ionic strength on the lytic activity of human skin lysozyme were the same as those on the lysozyme from human milk and hen egg white. The effects of preincubation

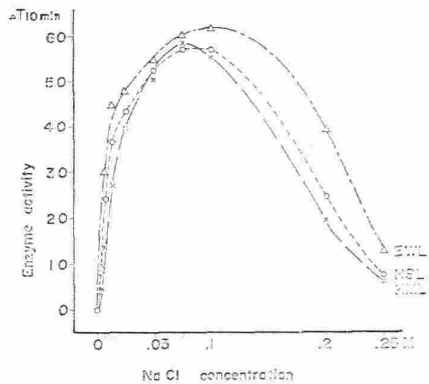

FIG. 5. Effect of sodium chloride on lysozyme activity. Abbreviations as in Fig. 4.

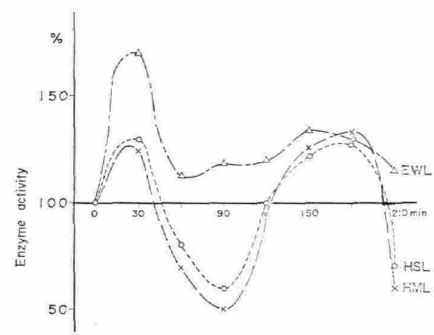

FIG. 6. Effect of preincubation of lysozyme activity. Abbreviations as in Fig. 4.

periods on the lytic activity of human skin lysozyme are quite similar to those of human milk lysozyme, but they are different from those of the hen egg white lysozyme.

It is of interest to find that the lysozyme content of human skin is almost identical with that of human saliva reported by Ogawa (3).

Klenha and Krs (2) reported on the content and properties of lysozymes in mouse skin homogenates and human surface extracts, and pointed out that lysozyme in human skin surface might come directly from skin rather than from the blood capillaries. They indicated the need to purify the enzyme for structural or immunochemical studies.

In the present studies of human skin lysozyme, it has been shown that the biochemical properties of human skin lysozyme are quite similar to those of human milk and leucocyte lysozymes. Therefore, it seems likely that the human lysozymes are not organ specific. Although lysozymes from various tissues appear to be similar biochemically, further immunochemical analysis of human lysozymes will be necessary to elucidate the fine structural relationships among them. These immunochemical studies of human lysozymes are in progress in our laboratory.

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REFERENCES

1. Liss, M.: Isolation of a heat-stable crystalline protein from psoriatic scales., Biochemistry, *4:* 2705, 1965.
2. Klenha, J. and Krs, V.: Lysozyme in mouse and human skin., J. Invest. Derm., *49:* 396, 1967.
3. Ogawa, H.: Lysozyme content of human skin. Jap. Jour. Derm., Series B, *81:* 1971.
4. Jollés, P. and Jollés, J.: Lysozyme from human milk. Nature, *192:* 1187, 1961.
5. Jollés, J. and Jollés, P.: Human tear and human milk lysozyme. Biochemistry, *6:* 411, 1967.
6. Petit, J. F. and Jollés, P.: Lysoyzme from human saliva. Nature, *200:* 168, 1963.
7. Petit, J. F., Panigel, M. and Jollés, P.: Purification et analyse duun lysozyme extrait du placenta humain. Bull. Soc. Chim. Biol., *45:* 211, 1963.
8. Charlemagne, D. and Jollés, P.: Les lysozyme des leucocytes et du plasma d'origine humaine. Nouvelle Revue Francaise d'Hamatologie, *6:* 355, 1966.
9. Osserman, E. F. and Lawlor, D. P.: Serum and urinary lysozyme (muramidase) in monocytic and monomvelocytic leukemia., J. Exp. Med., *124:* 921, 1966.
10. Kimura, M., Otaki, N., Ogawa, H., Murayama, K. and Kobayashi, S.: Ind. Health, *8:* 22, 1970.
11. Parry, R. M., Chandan, R. C. and Shahani, K. M.: A rapid and sensitive assay of muramidase. Proc. Soc. Exp. Biol. Med., *119:* 384, 1965.
12. Reisfeld, R. A., Lewis, U. J. and Williams, D. E: Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature, *195:* 281, 1962.
13. Parry, R. M., Chandan, R. C. and Shahani, K. M.: Isolation and characterization of human milk lysozyme. Arch. Biochem. Biophys., *130:* 59, 1969.